APPENDIX B

interactions. At 5 to 6 weeks, the animals turn gray because of apoptosis of melanocytes. The hematopoietic system is initially normal, but thymus and spleen subsequently undergo massive involution due to apoptosis, reflecting a failure to maintain homeostasis in both B and T cells. Mice lacking *Bcl-X*$_L$ are unable to complete normal development, with embryos dying of erythroid and neuronal apoptosis.[104,105] It thus appears that different antiapoptotic family members predominate in a tissue- and developmental-specific manner.

Loss of the proapoptotic gene *Bax* results in hyperplasia of thymocytes and B cells and accumulation of atrophic granulosa cells and excess primordial follicles that fail to undergo apoptosis.[106] The male mice are infertile because of failure of normal postnatal death of spermatogonia. This leads to a markedly disorganized seminiferous tubule and failure to successfully complete meiosis. Increased cell numbers are present in *Bax*-deficient neurons, indicating that cells that normally would have died during embryonic development because of inadequate innervation are saved in the absence of *Bax*.[107]

Mice without BID successfully complete embryonic development and appear grossly normal. However, the mice are resistant to Fas-induced hepatocellular apoptosis, indicating a critical role for a BID-dependent mitochondrial amplification loop in this Fas-signaled death.[108]

ROLE OF MITOCHONDRIA

The mitochondrial dysfunction that occurs in cell death manifests as an initial hyperpolarization, followed by a loss of $\Delta\psi m$; the release of proteins from the mitochondrial intermembrane space, such as cytochrome *c*; and altered mitochondrial physiology, including the production of reactive oxygen species. Prior studies of necrotic death and late stages of apoptotic cell death have noted mitochondrial swelling attributed to the opening of a mitochondrial permeability transition pore that allows the passage of solutes and dissipation of the transmembrane gradient. The localization of antiapoptotic molecules, such as *BCL-2* and *BCLX*$_L$, as well as the translocation of proapoptotic BAX and tBID to the mitochondrial membrane, emphasizes the importance of mitochondrial dysfunction in the action of these molecules. The specific mechanisms by which these proteins elicit their mitochondrial effects is an area of intense interest.

The importance of the mitochondria in the execution of apoptosis varies depending on both cell type and death stimulus. In certain cell types, activation of the TNF/Fas death receptor activates robust quantities of caspase 8 and subsequent effector caspase 3 with no requisite role for mitochondria, whereas other cells such as liver require the mitochondrial amplification loop to die. Other death stimuli, such as growth factor deprivation, may proceed in the absence of caspases and depend heavily on mitochondrial dysfunction.[109–111]

CELL PROLIFERATION AND APOPTOSIS

Apoptosis represents a brake on cellular expansion, countering abnormal cell proliferation. Substantial evidence exists for cross-talk between proliferation and apoptosis pathways.[112] The oncoproteins *c-Myc* and adenovirus E1A, both potent inducers of proliferation, also have been shown to possess proapoptotic properties.[113–116] The mitogenic and apoptotic properties of both *c-Myc* and adenovirus E1A are genetically inseparable.[113,117,118] E1A induces proliferation and apoptosis by interacting with either the retinoblastoma protein (Rb), a regulator of cell-cycle progression, or the transcriptional corepressor p300.[119–123] *c-Myc* appears to promote apoptosis by multiple pathways.[112]

Rb itself also provides a link between cell proliferation and apoptosis. Rb functions as a cell-cycle checkpoint between G_1 and S phase and mediates its effect through interaction with a family of transcription factors that control the expression of genes required for cell-cycle progression, the E2F proteins.[124–126] Complexes containing both E2Fs and Rb have been shown to bind to target DNA sequences in a number of promoters and actively repress transcription.[127–130] Entry into S phase induced by ectopic expression of E2F or mutagenesis, which abolishes interaction with Rb, results in increased apoptosis.[131–133] Mice in which the Rb gene has been knocked out by homologous recombination die at embryonic day 12 to 13 and exhibit both proliferation and apoptosis of liver, central nervous system, lens, and skeletal muscle.[134,135] E2F-1 knockout mice develop a broad spectrum of tumors, including lymphomas, and display decreased apoptosis in double-positive thymocytes, further establishing the link between cell proliferation, apoptosis, and tumorigenesis.[136,137]

Oncogenes have been shown to sensitize cells to a wide variety of stimuli, including DNA damage, hypoxia, death receptors such as TNF-α and Fas, and growth factor withdrawal.[113,138–144] It appears that the cellular machinery directing cell proliferation and apoptosis is coupled, suggesting that the decision of a cell to undergo apoptosis or proliferation may be determined by the balance between growth and survival signals.[145]

One potential link between these two processes is the p53 tumor suppressor. Loss of p53 has been observed in numerous tumor types, and p53 function is abrogated in a large percentage of tumors.[146,147] p53 expression is induced in response to a variety of cellular stresses, including DNA damage, hypoxia, and oncogene activation, resulting in cell-cycle arrest or apoptosis. Mice deficient for p53 are developmentally normal, but 75% develop spontaneous tumors by 6 months of age.[148] Germline mutation of p53 in humans results in Li-Fraumeni syndrome, and more than 50% of these individuals develop tumors by 30 years of age.[149]

The majority of p53 mutations in human tumors cluster within the DNA-binding domain, suggesting that p53 exerts its tumor suppressor effects through transcriptional regulation of target genes.[150] The mechanism by which p53 exerts its apoptotic effect appears to be multifactorial. p53 is able to induce the expression of *BAX* and *FAS*, as well as another member of the TNF family of death receptors, DR5.[151–154] In addition, p53 inhibits the expression of *BCL-2*, and BCL-2 can inhibit p53-induced apoptosis in select settings.[155–158] p53 also appears to induce apoptosis by post-translational mechanisms.[159,160]

POSSIBILITIES FOR THERAPEUTIC INTERVENTION

Given the ability to induce apoptosis in lymphoid cells and many types of tumor cells, the death receptors are attractive targets for therapeutic intervention in cancer. However, infusion

of TNF-α causes a lethal inflammatory response resembling septic shock, which results from proinflammatory activation of macrophages and endothelial cells,[161,162] and infusion of agonistic anti-Fas antibody causes lethal hepatic apoptosis. The related death ligand TRAIL (APO2L) appears to possess the ability to induce apoptosis in a wide variety of tumor cell lines. *In vivo* administration of a leucine zipper form of TRAIL in which the molecule is stabilized as a trimer suppresses the growth of a mammary adenocarcinoma cell line in SCID (severe combined immunodeficiency) mice.[163] Normal cells treated *in vitro* with TRAIL showed no decreased viability. Similarly, recombinant TRAIL administered shortly after tumor xenograft injection markedly reduces tumor incidence. In addition, treatment of mice bearing solid tumors resulted in tumor cell apoptosis as well as improved survival. A synergistic effect was obtained with TRAIL and 5-fluorouracil or irinotecan (CPT-11). Encouragingly, intravenous injections of TRAIL into nonhuman primates did not result in toxicity to tissues or organs.

The *BCL-2* gene provides another promising target for therapeutic intervention, particularly in the therapy of low-grade lymphoma in which BCL-2 overexpression plays an important role.[164] The strategy of antisense oligonucleotide therapy has been used to "silence" *BCL-2* expression. Antisense oligonucleotides are short stretches of DNA, approximately 16 to 20 bases in length. The oligonucleotides are internalized by cells through a saturable endocytosis pathway. On injection into a host, expression of a specific gene can be blocked by hybridization with the target messenger RNA through Watson-Crick base pairing. The result is either degradation of the RNA-DNA complex by Rnase H or block in translation of the RNA.

An 18-base-pair antisense oligonucleotide, G3139 (Genta, San Diego, CA), was designed against *Bcl-2* for the treatment of follicular lymphoma.[165] Initial studies in a t(14;18) murine xenograft lymphoma model were encouraging, with absence of disease by polymerase chain reaction in 10 of 12 animals tested. A phase I clinical trial of G3139 has been completed on patients with relapsed B-cell non-Hodgkin's lymphoma with evidence of BCL-2 overexpression by immunohistochemistry of lymph node biopsy.[166] The main toxicity was reversible thrombocytopenia. Of the 20 evaluable patients (N = 21), one complete response was achieved in a patient with stage IV follicular lymphoma. Two patients had partial responses, eight patients had stable disease, and nine patients progressed. Current phase II studies are under way to investigate the role of G3139 in combination with conventional chemotherapy.

BCL-2 also has been shown to play a role in solid tumors. In prostate cancer, Bcl-2 overexpression confers both chemoresistance and resistance to apoptotic cell death after androgen withdrawal.[167–172] In an androgen-dependent tumor model, *in vitro* treatment of tumor cells with antisense *BCL-2* enhances cytotoxicity of paclitaxel.[167] *In vivo* administration of antisense *BCL-2* oligonucleotides in combination with paclitaxel to animals with established tumors results in inhibition of tumor growth. In addition, treatment in combination with paclitaxel after castration results in a significant delay in tumor recurrence. BCL-2 is also highly expressed in malignant melanoma.[173,174] In a preclinical xenograft model, *BCL-2* antisense oligonucleotides significantly sensitized the tumor cell response to subsequent dacarbazine.[175] It thus appears that *BCL-2* antisense therapy may have a potential role in combination with other chemotherapeutic drugs as a chemosensitizing agent.

CONCLUSIONS

Apoptosis is an evolutionarily conserved, highly regulated mechanism for maintaining homeostasis in multicellular organisms. Numerous signals are capable of modulating cell death. After a death stimulus, the signal is propagated and amplified through the activation by proteolytic cleavage of caspases, culminating in the ordered disassembly of the cell. The process may transpire through a mitochondrial-dependent or -independent pathway, depending on the death signal and cell type involved. The Bcl-2 family of proteins is situated upstream of irreversible cell damage in the apoptotic pathway, providing a pivotal checkpoint in the fate of a cell after a death stimulus. The proapoptotic molecules BID, BAD, and BAX undergo modification and intracellular translocation on receipt of a death stimulus, connecting distinct upstream signal transduction pathways with the common, core apoptotic pathway. The distribution of inactive conformers of the BH3-only members suggests that they may function as sentinels for recognizing cellular damage.[66] BIM would monitor microtubule function, BID would amplify minimal caspase 8 activation, and BAD would patrol for metabolic stress after loss of critical survival factors. This model would explain how seemingly diverse cellular injuries converge on a final common pathway of cell death.

Finally, the cellular pathway to apoptosis appears to communicate with the pathway for cell proliferation.[112] As a result, activation of cell proliferation by oncogenes also results in sensitization to apoptosis. Reciprocally, the expression of antiapoptotic molecules often retards cell-cycle progression.[176] This interconnection provides a means for limiting the threatening expansion of cells with a lesion in either pathway. These observations fit the evidence that defects are required in both proliferation and cell death pathways, as single defects tend to be self-correcting in their net effect on cell number. The molecules mediating apoptotic pathways provide an exciting opportunity for rational design of new therapeutic agents to specifically promote apoptosis of cancer cells.

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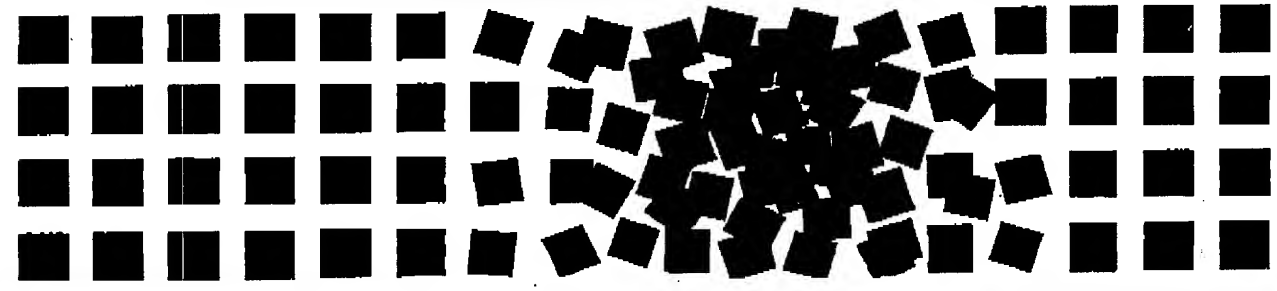

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